Effects of Age, Ambient Temperature, and Heat-Stable *Escherichia coli* Enterotoxin on Intestinal Transit in Infant Mice

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Some interrelationships among age, ambient temperature, intestinal transit, and enterotoxigenic *Escherichia coli* infection were studied in an infant mouse model. The transit of dye in the small intestine was accelerated during the response to heat-stable *E. coli* enterotoxin. Transit in the small intestine of normal mice accelerated with increased age (from <17 h to 8 days old) and accelerated with increased ambient temperature (from 25 to 37°C). Transit was more rapid in the jejunum than in the ileum throughout the range of experimental conditions studied. *E. coli* strains that do not produce any of the pili known to facilitate intestinal colonization were cleared from the small intestine more rapidly at 37°C than at 25°C. This clearance was thought to be due to accelerated transit at the higher temperature. In contrast, a strain of *E. coli* that produces K99 (pili previously shown to facilitate intestinal colonization in other species) was not cleared from the small intestine and colonized more intensively at 37°C than at 25°C. Intensified colonization by this strain was thought to be due to increased production of K99 at the higher temperature. It was suggested that sluggish intestinal transit may also be characteristic of the neonates of other species and be one of the factors predisposing them to intestinal colonization by enteropathogens. It was speculated that this predisposition may be enhanced if the neonates are chilled. However, the effect of ambient temperature on intestinal transit in homeothermic neonates such as pigs, calves, and humans may be different from that in mice because neonatal mice are poikilothermic.

There are several lines of evidence consistent with the hypothesis that transit in mammalian small intestine is sluggish at birth, accelerates with age, and is influenced by ambient temperature. Neonates are born germfree, and transit in the small intestine of germfree mice is slower than in conventional mice (1). Intestinal transit in rats accelerates with age (26). Motility tends to protect the small intestine from bacterial colonization (7, 12, 24), and the small intestine of neonates is more readily colonized by some bacteria than that of adults (9, 10, 15, 21, 22). Diarrheal disease caused by enterotoxigenic *Escherichia coli* (ETEC) is dependent on colonization of small intestine, and in animals the disease has a marked predisposition to occur during the early neonatal period (16, 21, 22). The thermoregulatory abilities of pigs and mice are poorly developed at birth (2, 6), and chilling is generally considered to predispose pigs to diarrheal disease caused by ETEC (3, 11) and by enteropathogenic viruses (20).

We reported that the character of the re-

sponse of infant mice to heat-stable *E. coli* enterotoxin (ST) varied with age and ambient temperature, and suggested that these differences in response are due to accelerated transit in the small intestine with age and temperature (14). There is also reason to think that enterotoxins alter intestinal transit during diarrheal disease caused by ETEC. For example, *Vibrio cholerae* enterotoxin and *E. coli* enterotoxins stimulated intestinal contractions (5, 13; M. C. Goldschmidt, N. W. Weisbrodt, and J. Walther, Abstr. Annu. Meet. Am. Soc. Microbiol. 1976, B69, p. 22). In contrast, ST depressed intestinal contractions in vitro (18), and one group found that infant mice exposed to ST had impaired gastric emptying and accumulated fluid in their stomachs (23).

We have attempted to use infant mice as a model for the study of the effects of ambient temperature on intestinal transit and ETEC infections in neonates. The objectives of the work reported here were: first, to see if intestinal transit in infant mice changes with age, ambient

temperature, and ST exposure; second, to see if ambient temperature affects intestinal colonization of infant mice by *E. coli*; and third, to attempt to confirm the report that ST induces gastric fluid accumulation and impaired gastric emptying in infant mice.

MATERIALS AND METHODS

Infant CF1 mice from ARS Sprague Dawley, Madison, Wis., were weaned at ages from <17 h to 8 days. One-half hour after weaning, they were placed in egg incubators (Favorite Incubators, model 624-E; Leahy Manufacturing Co., Higginsville, Mo.) at 20°C and 70% relative humidity, at 25°C and 78% relative humidity, at 30°C and 69% relative humidity, or at 37°C and 66% relative humidity. The incubators contained wooden boxes divided into compartments (7 cm square and 5 cm deep). The floor of each compartment was covered with a sheet of white filter paper. Incubator fans ran constantly, and mice were held one to a compartment until they were killed. After the mice had been in the incubators for 0.5 h, they were given either 1 drop of Evans blue dye (5% in distilled water) orally, or 0.1 ml, containing 10^6 viable *E. coli,* or ST, or negative control material, intragastrically via stomach tube. Mice given ST or control material were given 1 drop of Evans blue dye 15 min later. The dye was used as an indicator of intestinal transit. At intervals after dye exposure, mice were killed by exposure to chloroform, their gastrointestinal tracts were removed and unraveled, and the distances from pylorus to the leading edge of the dye marker and to the ileocecal valves were measured. In some experiments, observations on diarrhea, weight loss, and the ratios of weight of the intestinal tracts or stomachs to the weights of the remainder of the carcasses were recorded.

Mice were given 10^6 viable stationary-phase *E. coli* intragastrically and were killed 12 h later. The entire small intestine was immediately removed, and the total number of viable *E. coli* in small intestine was determined (16). The strains of *E. coli* used were originally isolated from the intestinal tracts of pigs. Strain 123 (O43:K–:H28) is non-enterotoxigenic, and strains 431 (O101:K30,99:NM) and 2176E8 (O138:K81) produce ST but not heat-labile enterotoxin. Strain 431 produces pili (K99 antigen), which facilitate colonization in the small intestines of pigs, calves, lambs, and possibly mice (8, 10, 17).

Enterotoxin and control material were prepared by inoculating 0.5 ml of stationary-phase Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) cultures into Erlenmeyer flasks (2-liter) containing 400 ml of Trypticase soy broth [pH 8.0 with 0.01 M tris(hydroxymethyl)aminomethane buffer and 0.013% Antifoam 60 (General Electric Co.)]. The flasks were sealed with cotton plugs and incubated aerobically at 37°C for 20 h with continuous shaking (200 cycles per min); then the bacteria were removed by centrifugation (12,000 × *g* for 10 min at 4°C), and the supernatant fluid was sterilized by filtration through 0.22-μm filter disks (Millipore Co., Bedford, Mass.) and stored at 4°C until used as ST or control material as described previously (14). ST was produced by *E. coli* 2176E8;

control material was produced from strain 123. ST and control preparations were given as 1:4 dilutions because the ST preparation elicited strong but not maximal fluid secretion at this dilution.

RESULTS

In initial experiments, mice <17 h and 2, 4, 6, or 8 days old were held at 25, 30, or 37°C, and three mice per age per temperature were examined at each 0.5-h interval from 0 to 4 h after they were given dye. There was dye in the stomachs of all mice killed at time 0, and in some mice at this time (apparently irregardless of age or temperature) dye extended up to 0.5 cm into the proximal small intestine.

Effect of age. Transit in small intestine tended to accelerate with age from <17 h to 8 days old (Fig. 1). This acceleration was also apparent at the other two temperatures (data not shown), in spite of large variations among individuals. Analysis of variance with all temperatures combined indicated that acceleration with age was significant, and othogonal polynomials indicated that this acceleration was chiefly linear ($P < 0.01$). An experiment was done to see if acceleration with age extended beyond 8 days. Mice 2.5 months old (from the same source and strain as the neonates) were held at 25°C and given dye, and transit was recorded at 0.5-h intervals (three mice per interval) as usual. The earliest observed time for dye to reach the cecum of an individual in this group was 1.5 h. This contrasted with the previous experiment on 25°C, 8-day-old mice, in which cecal dye was not observed until 2.5 h, and indicated that transit in small intestine is more rapid at 2.5 months than at 8 days of age.

Effect of temperature. Transit also tended to accelerate with temperature between 25 and 37°C (Fig. 2). This tendency was also apparent in the <17-h, 4-day, 6-day, and 8-day age groups (data not shown). In these initial experiments acceleration was only marginally significant statistically ($P = 0.10$, analysis of variance with all ages combined, 25 to 37°C), and transit in 2-day-old mice appeared to be the same at 30 and 37°C. Three additional replicates with 2-day-old mice at 30 and 37°C were done. When all replicates were combined, transit was significantly faster in the 37°C mice (analysis of variance with log transformation, $P = 0.02$). This difference was apparent by the time the dye had reached the distal small intestine. Additional replicates confirmed the persistence of acceleration with temperature in 8-day-old mice (Fig. 3).

The numbers of viable *E. coli* of strains 123 and 2176E8 recovered from small intestine 12 h after exposure decreased significantly ($P < 0.05$,
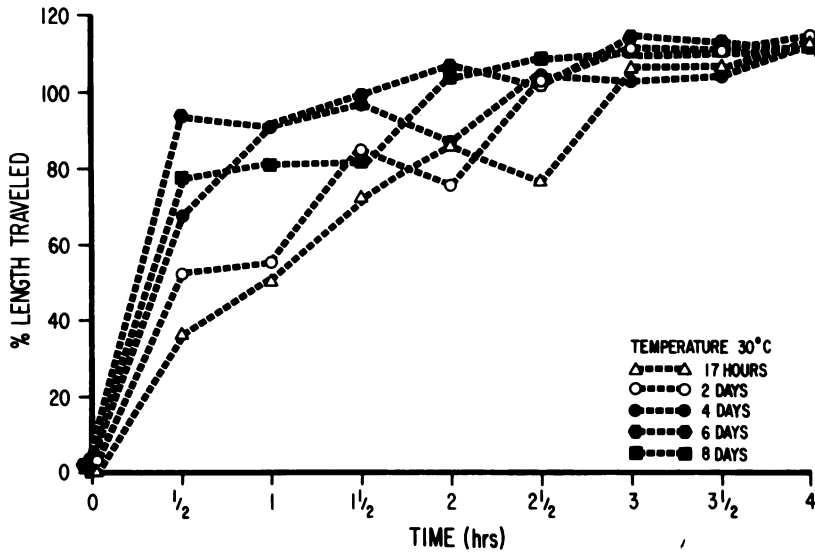
FIG. 1. *The percent length of small intestine traveled by dye, at intervals after mice were given Evans blue dye orally. Values >100% indicate that the leading edge of the dye was in the large intestine. Mice were from <17 h to 8 days old and held at an ambient temperature of 30°C. Mean of three mice per data point.*
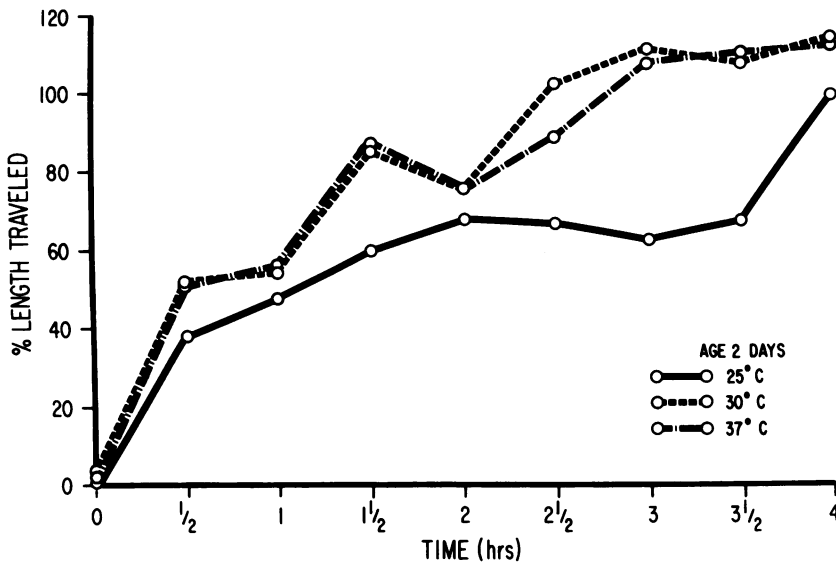

FIG. 2. *The percent length of small intestine traveled by dye, at intervals after mice were given dye orally. Two-day-old mice were held at an ambient temperature of 25, 30, or 37°C. Mean of three mice per data point.*

analysis of variance) from 25 to 37°C, as expected (Fig. 4). However, the numbers of strain 431 increased significantly ($P < 0.001$) over this temperature range. In view of this difference between strains, the experiment was expanded to include mice held at 20°C. All three strains tended to attain greater numbers in the 20°C mice than they had in the 25°C mice (Fig. 4).

Effect of site. Transit tended to be more rapid in proximal than in distal small intestine

(Fig. 1 to 3). For example, in 2-day-old, 30°C mice, the leading edge of the dye traveled the proximal one-half of the small intestine in about 0.5 h, whereas an additional 2 h was required for travel through the distal one-half of the small intestine (Fig. 1 and 2). At each age and temperature, the percentage of small intestine traveled during the first 30 min was significantly ($P < 0.01$, analysis of variance followed by contrast) greater than that calculated for the last 30-min

period before dye was first observed in the cecum of an individual at that age and temperature.

Effect of ST. Transit in the small intestine of 37°C, 2-day-old mice exposed to ST was more rapid than in controls, and ST exposure did not inhibit the movement of dye from stomach into small intestine (Fig. 5). There was fluid in the intestines of ST-exposed mice (mean intestine weight/body weight = 0.104 for ST-exposed and
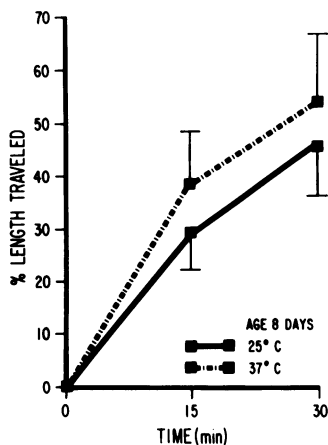

FIG. 3. *The percent length of small intestine traveled by dye, at intervals after mice were given dye orally. Eight-day-old mice were held at an ambient temperature of 25 or 37°C. Mean and standard deviation of 12 mice per data point. Analysis of variance indicated that transit at 37°C was 2 to 14% faster than at 25°C (95% confidence interval).*
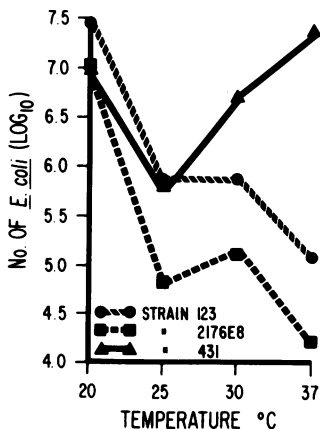

FIG. 4. *Numbers of viable E. coli recovered from the small intestine of mice held at different ambient temperatures for 12 h after intragastric inoculation with 10^6 E. coli per mouse. E. coli strain 123 is serotype O43:K–:H28, strain 2176E8 is O138:K81, and strain 431 is O101:K30,99:NM. Geometric mean of 12 mice per data point.*
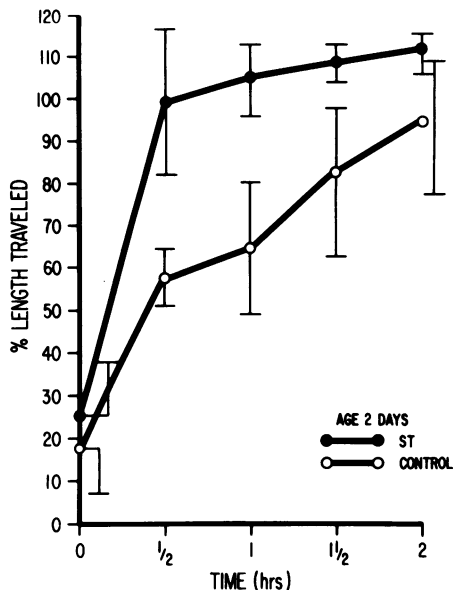

FIG. 5. *The percent length of small intestine traveled by dye, at intervals after mice were given dye orally. Two-day-old mice, held at an ambient temperature of 37°C, were given (intragastrically) E. coli ST or material from a non-enterotoxigenic strain of E. coli (control) 15 min before they were given dye. Mean and standard deviation of 12 mice per data point. The percent length traveled by 1 h was significantly greater (P < 0.001, t test) for ST mice than for controls.*

0.063 for controls) at 0.5 h (45 min after ST exposure). All ST-exposed mice had diarrhea and had excreted dye in feces by 2 h. None of the controls had diarrhea or excreted dye. None of the stomachs in either group was distended with fluid at any time, and some dye and milk persisted in the stomachs of all mice throughout the experiment. The report of ST-induced impaired gastric emptying and gastric distention dealt with mice held at 22°C (23). When our experiment was replicated at 22°C, we again observed accelerated transit in ST-exposed small intestine (Fig. 6). None of the mice in either group experienced gastric distention, and the stomach weight/carcass weight ratios of ST-exposed and control mice were similar (Fig. 6).

DISCUSSION

Accelerated transit after exposure to ST could be the result of a direct effect of ST on nerves or muscle, stimulating contractions of the intestine or relaxation of the ileocecal valve. Our results with ST are consistent with previous reports that cholera enterotoxin, heat-labile *E. coli* enterotoxin, and ST directly stimulate intestinal contractions (5, 13; Goldschmidt et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1976, B69, p.
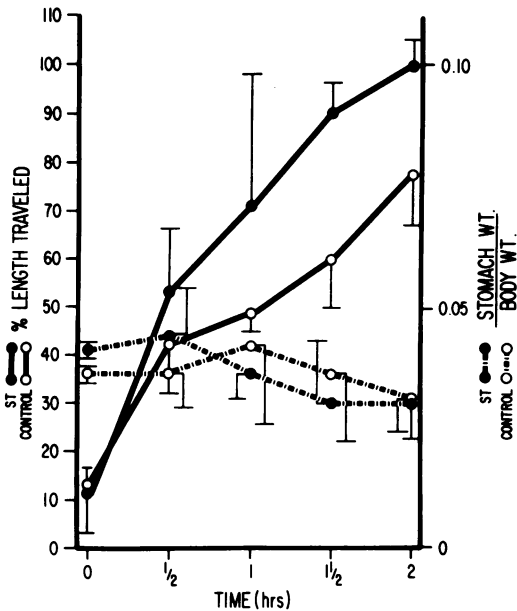
FIG. 6. *The ratio of stomach weight to body weight and the percent length of small intestine traveled by dye, at intervals after mice were given ST intragastrically and dye orally. Two-day-old mice, held at an ambient temperature of 22°C, were given ST 15 min before they were given dye. Mean and standard deviation of 12 mice per data point. The percent length traveled by 1 h was significantly greater (P = 0.038, t test) for ST mice than for controls.*

22). However, it also seems likely that at least part of the ST-induced accelerated transit reported here was due to a decrease in viscosity and an increase in volume of luminal contents, as the result of intestinal secretion of fluid. We were unable to confirm the report of Stavric and Jeffrey (23) that ST induces gastric fluid accumulation or impairs gastric emptying.

Transit in the small intestine of normal mice was comparatively slow during the neonatal period and even more so when the neonates were held at low ambient temperatures. Furthermore, as in other species and ages (25), transit was slower in ileum than in jejunum. Slow transit would be expected to predispose the small intestine to colonization by infectious agents. For example, it may contribute to the neonatal predisposition of pigs, calves, and mice to enteric diseases that are dependent on colonization of the small intestine by pathogens such as ETEC (9, 10, 21, 22). It may render neonates even more susceptible to such diseases if they are chilled. Some of the ETEC that colonize pig or calf small intestine by adhesion to the mucosa will colonize ileum but not jejunum even though they can adhere to mucosa at both sites (8, 16). If other

factors are constant, then transit is directly related to contractions in small intestine (19). Thus, comparatively sluggish motility (transit, peristaltic contractions, segmental contractions, and contractions of villi) in ileum may explain why these ETEC colonize ileum but not jejunum.

The thermoregulatory abilities of neonatal mammals are generally less well developed than those of adults; however, there are marked differences among species (2). Neonatal mice are essentially poikilothermic but develop thermoregulatory abilities with age (apparently over several weeks), and adults tend to maintain their body temperature at about 37°C (2, 4). Thus, the effects of ambient temperature on intestinal *E. coli* (Fig. 4) were probably the result of differences among groups of mice in intestinal temperature as well as transit. There tended to be fewer of strains 123 and 2176E8 in intestine at the higher ambient temperatures. Presumably accelerated transit at higher ambient temperatures was the predominant effect on these two strains. This probably also explains why the number of strain 431 organisms present in 25°C mice tended to be lower than in 20°C mice. In contrast, strain 431 increased in number with temperatures above 25°C. That is, at higher temperatures this strain apparently overcame the tendency for accelerated transit to clear it from the intestine. We think the ability of strain 431 to overcome clearance at higher temperature was related to its production of K99. K99 is produced at 37°C but not at 18°C (17). In pigs, calves, and lambs, K99 facilitates intestinal colonization by mediating the adhesion of carrier strains to the mucosa. In preliminary studies, strain 431 formed layers of adherent bacteria on villi in the small intestines of infant mice (2 to 4 days old, ambient temperature about 25°C), but the K99-negative strains 123 and 2176E8 did not (B. Nagy and H. W. Moon, unpublished data). Thus, K99 apparently also facilitates intestinal colonization by adhesion in infant mice.

The existence and complexity of the interactions among age, ambient temperature, intestinal transit, and intestinal colonization reported here should be considered in design and interpretation of studies of enteric infections in infant mice. More definitive study of these interactions in mice may also yield some insight into such interactions in enteric disease in the newborn of other species. However, in contrast to mice, neonatal pigs, calves, lambs, and humans are homothermic and tend to maintain constant body temperatures via expenditure of metabolic energy and behavioral adaptations (2). The pig's capacity to do so at birth is less than that of

calves and lambs but increases markedly by 2 days of age (6). The body temperature of neonatal pigs does decrease at ambient temperatures of 5 to 10°C or less (6). Furthermore, there appear to be adverse effects (cold stress) associated with the prolonged summit or near-summit metabolism resulting when ambient temperatures are less than optimal but still high enough to permit homothermic neonates to maintain body temperature (2, 6).

The mechanism(s) of the effect of ambient temperature on transit in neonatal mice is unknown. It seems reasonable that it may just be one direct effect of the general increase or decrease in metabolism with increased or decreased body temperature in these poikilotherms. If so, homothermic neonates would tolerate much lower ambient temperatures than mice, before decreased transit would occur (25 to 30°C for mice versus 5 to 10°C for pigs). On the other hand, if decreased transit results from a more indirect effect of adaptation to cold stress, then it would be expected to occur in homothermic neonates at suboptimal ambient temperatures which still permit maintenance of body temperature. These alternatives are not mutually exclusive.

It is also conceivable that much of the effect of age on transit in mice reported here reflected increased thermoregulatory ability of mice during the first 8 days of life. If so, this was not the sole explanation for the effect of age on transit, because transit accelerated with age even when the mice were held at 37°C. Studies on the effects of age and ambient temperature on transit in homothermic neonates are warranted.

ACKNOWLEDGMENTS

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